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Radiation and Chemical Activation of *ras* Oncogenes in Different Mouse Strains

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A survey of a large series of radiation- or chemically induced thymic lymphomas in (AKR X RF)F_1, RF/J, 129/J, and C57BL/6J mouse strains for activated *ras* oncogenes showed that of the tumors containing transforming activity, in more than 75% of the cases this activity segregated with either K-*ras* or the N-*ras* gene. H-*ras* activitiy was never detected. The genetic background of the host influenced susceptibility to tumor induction and oncogene activation. The K-*ras* gene was preferentially activated over the N-*ras* gene (approximately 2:1) whether the inducing agent was radiation or the chemical *N*-nitrosomethylurea. The activating mutation for the K-*ras* gene was consistently identified as a GGT to GAT transition in codon 12. In contrast, several different mutations of the N-*ras* gene were identified and localized to codons 12, 13, or 61. Assessment of the allelic composition of the *ras* locus shows that some proportion of the tumors lost the normal *ras* allele.

Introduction

Induction of genetic alterations in DNA of mammals by carcinogenic agents has long been a useful model system for understanding the events in cancer development. Somatic mutations of specific cellular genes are known to occur in human tumors and in a variety of experimental animal tumors. Cellular genes belonging to the *ras* gene family have been repeatedly identified in a significant proportion of these human and animal tumors (*1,2*).

The *ras* gene family is comprised of three genes that have homology in their DNA sequences. Two members were identified in the oncogenic Harvey (H-*ras*) and Kirsten (K-*ras*) rat sarcoma viruses. The third member of the family, N-*ras*, was isolated from a human neuroblastoma. This family of genes encodes a protein known as p21 due to its mobility upon gel electrophoresis. It is highly conserved in evolution and is normally expressed in mammals in a tissue-specific manner (*3,4*).

The function of the p21 proteins is largely unknown. However, the fact that they are so highly conserved in evolution implies an important role in cell growth or differentiation or both. The present evidence is for a role in signal transduction (*1*). The *ras* proteins are located at the inner surface of the cell membrane. Here they may act as messengers to transmit signals received from outside the cell from hormones and growth factors to inside the cell where the signal is translated as a message for cell division.

The mechanism of carcinogen-induced oncogene activation is not understood. The genetic lesions that result in activation of the *ras* cellular gene are single point mutations. The presence of the mutation leads to a substitution of amino acids that have been identified at positions 12, 13, 61, or 117 of the p21 protein (*1,5,6*).

Experimental Animal Model of Tumor Induction

In an attempt to understand some of the events involved in carcinogen-induced tumors in man, we have chosen to study tumor induction in inbred strains of mice. Ionizing radiation, neutron radiation, and the DNA alkylating agent *N*-nitrosomethylurea (NMU), all known to produce tumors in experimental animals, were chosen for study. The inbred strains RF/J, C57BL/6, 129J, BALB/c, and the F_1 hybrid between AKR/J and RF/J used in these studies each provide different genetic back-

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grounds to determine the influence of the host on tumor induction by the different agents.

Thymic lymphomas were induced in these strains of mice with high frequency using well-defined treatment protocols. Ionizing gamma radiation was given according to the Kaplan protocol of 4 weekly doses of 150 to 175 rads/dose beginning at 4 weeks of age (*7*). High LET ionizing neutron radiation was used to treat 4-week-old RF/J female mice once with 100 rads of 0.4 MeV neutrons. NMU treatment consisted of 5 weekly injections IP (30 mg/kg) of 4-week-old animals (*8*).

Several questions concerning tumor initiation and progression can be addressed using animal model systems. Use of genetically identical mice of different strains treated with the same carcinogenic agents will allow one to determine whether the genetic background of the host influences oncogene activation. A second question that is of interest is whether treatment of the same strain of mice with different carcinogenic agents produces activating mutations that are carcinogen specific. Last, since cancer development is believed to be a multistage process, we would like to determine at what stage of tumor development *ras* genes are involved.

Methods for Detecting Transforming DNA Sequences

The ability to detect transforming DNA sequences or activated oncogenes is essential to the success of studying their role(s) in tumor development. The biological activity of oncogenes is measured in assays where tumor-derived DNA is introduced into mouse fibroblasts. The presence of a transforming DNA sequence is scored by a change in morphology of the mouse fibroblast after gene transfer by focus formation in 5% calf serum. Under these conditions of selection, transformed cells have a growth advantage over normal cells.

Another method that is able to detect transforming genes that lack the ability to induce focus formation in mouse fibroblasts is outlined in Figure 1. Tumor DNA is cotransferred with a dominant selectable biochemical marker. Cells that take up the foreign DNA will survive in the presence of the antibiotic G418 and form colonies. After 2 weeks, the colonies are harvested for inoculation into nude mice. Tumor formation is associated with the presence of transforming DNA sequences (*9*).

The transformation arising in either the focus assay or the tumorigenicity assay are then subjected to molecular analysis to characterize the oncogene (*ras* or non-*ras*) and its activating mutation. Because the DNA gene transfers were intraspecific, positive 3T3 transformants were screened by Southern blot analysis for the presence of DNA rearrangements which occur when transfected DNA sequences integrate into the NIH 3T3 genome. The *ras* genes are detected in high frequency in these 3T3 transformants as additional DNA bands over the endogenous *ras* germline pattern. Amplification of the acquired *ras* sequences is frequently observed in positive transformants.

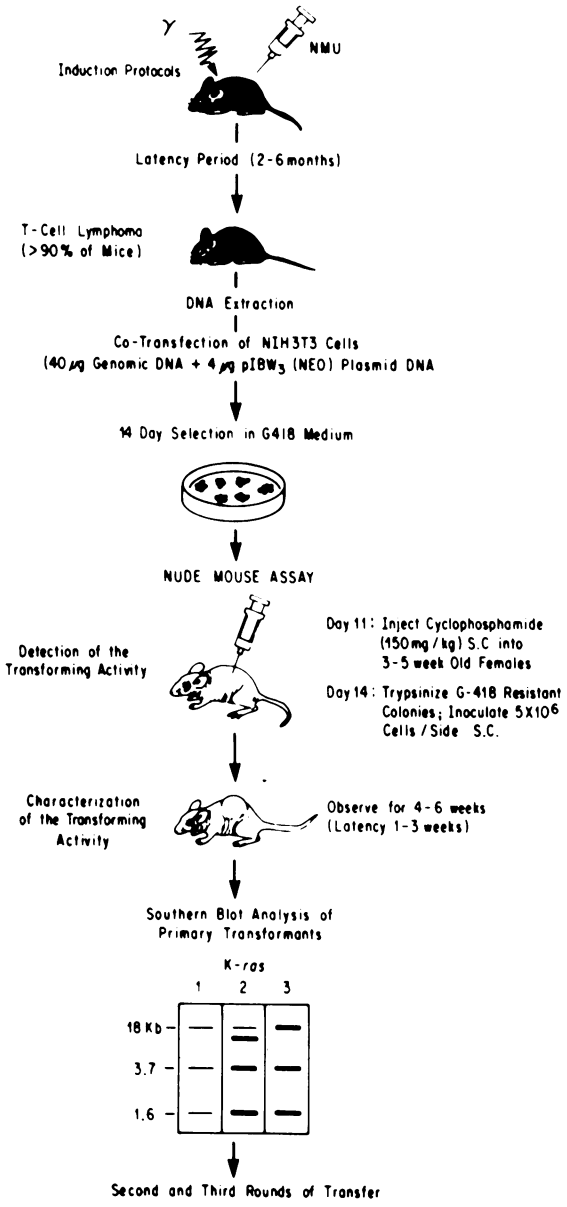

FIGURE 1. Detection of transforming activity in DNAs from carcinogen-induced thymic lymphomas by the tumorigenicity assay.

Methods for Identifying Activating *ras* Mutations

The genetic lesions that activate *ras* genes have been identified as single point mutations in each of the *ras* genes at similar sites. The codons 12, 13, or 61 are most frequently involved in this family of oncogenes. Three methods have been used in these studies to identify the activating mutations in the K- and N-*ras* genes characterized by Southern blot analysis. Two N-*ras* positive transformants were cloned, and the *ras* DNA fragments were sequenced to determine the activating mutations (*10*). The presence of certain mutations is known to change a restriction enzyme site, thus creating DNA fragment polymorphisms that are easily detected in

Southern blots. The most widely used method has been oligonucleotide mismatch hybridization (*11*). This method is based on the difference in melting temperature of a perfectly matched DNA—DNA hybrid relative to that of a hybrid with a single base pair mismatch. Synthetic oligonucleotides of 19 bases complementary to the normal 12th, 13th, or 61st codon of the N-*ras* gene and to the normal 12th or 61st codon of the K-*ras* gene were prepared. In addition, oligomers of 19 bases were prepared for all possible mutations of these codons, each with a base substitution at the middle position #10 of the oligomer. The mutations were first identified in the NIH 3T3 transformants and then in the primary lymphoma which served as the original source of the transforming DNA.

Results

Tumor Induction and Detection of Transforming Activity

The inbred strains RF/J, 129/J, C57BL/6J, and the F_1 hybrids of (AKR/J × RF/J) mice were treated with the Kaplan protocol of four fractionated doses of gamma radiation or with five NMU injections at weekly intervals. Thymic lymphomas were induced with high frequency in all groups of mice by both carcinogenic treatments with only one exception. 129/J mice were resistant to tumor induction by gamma radiation but highly susceptible to thymic lymphoma induction by NMU treatment.

Thymic lymphomas have also been induced in RF/J mice by neutron irradiation. The frequency (65%) was somewhat lower than that observed for radiation-induced tumors (>90%). The latent period of 6 months, however, was similar for both radiation-induced tumors in this strain.

Tumor DNAs were screened for transforming activity in NIH 3T3 cells using the calcium-phospate method of DNA-mediated gene transfer (*12*). Either the focus assay or the nude mouse tumorigenicity assay (Fig. 1) was used. DNAs from carcinogen-induced thymic lymphomas of different mouse strains varied in overall transforming activity. Approximately 35% of the thymic lymphoma DNAs from RF/J and 129/J mice were active in the focus-forming assay (*10*). With the same assay, 60 to 80% of carcinogen-induced tumors of (AKR times RF)F_1 mice produced 3T3 transformants (*13*). Tumors induced in C57BL/6J mice were analyzed in parallel in both assays. The nude mouse assay detected transforming activity in 80% of both groups of carcinogen-induced tumors, whereas the focus assay was less sensitive. None of the DNAs tested from radiation-induced thymic lymphomas of C57BL/6J mice produced foci whereas 50% of the DNAs from NMU-induced tumors were active (*14*).

Identification of the Carcinogen-Activated Oncogene and Its Distribution

Initially the DNAs from the 3T3 transformants were screened for *ras* sequences (H-, N-, and K-*ras*), as these oncogenes are most frequently detected in this assay. A total of 58 tumors from all four strains of mice produced 3T3 transformants. Of these, 78% had transforming activity that segregated with either the K-*ras* (28/58) or the N-*ras* (17/58) gene. In no instance has H-*ras* activity been detected in any carcinogen-induced thymic lymphomas.

The remaining 22% (13/58) of the transformants did not appear to be positive for any activated ras gene by Southern blot analysis and p21 protein analysis. Preliminary screening of these transformants with a panel of known onocogene probes shows that they are negative for *myb*, *myc*, *neu*, and *raf*.

The frequency distribution of activated *ras* oncogenes in DNAs from NMU- or radiation-induced thymic lymphomas of different mouse strains is summarized in Table 1. The K-*ras* gene was preferentially activated, being present in 50% of the DNAs from carcinogen-induced tumors. Activation of the N-*ras* oncogene was observed in 29% of the tumor DNAs. The non-*ras* transforming activity present in the last group of transformants remains to be identified.

The results of this survey would suggest that host genes can influence the frequency of *ras* gene activation as well as the type of *ras* gene, which becomes activated as a result of carcinogen treatment. NMU treatment of 129/J or RF/J mice produced activation of K- and N-*ras* genes with similar frequencies. In contrast, C57BL/6 or (AKR × RF)F_1 mice treated similarly with NMU show preferential activation of K-*ras* or N-*ras* genes, respectively. Likewise, radiation treatment of RF/J and C57BL/6 mice produces tumors in which the K- and N-*ras* genes are activated in equal proportions. On the other hand, the K-*ras* gene is preferentially activated in (AKR × RF)F_1 mice following radiation treatment.

Table 1. Frequency distribution of *ras* oncogene activation in carcinogen-induced thymic lymphomas in different mouse strains.

Strain of mouse	Treatment of animal	Characterization of oncogenes[a]		
		K-*ras*	N-*ras*	Non-*ras*[b]
(AKR × RF)F_1	γ-rays	++++[c]	−	−
	N-methylnitrosourea	−	++++	−
RF/J	γ-rays	++	+	++
	N-methylnitrosourea	+++	++	+
C57BL/6J	γ-rays	+	++	++
	N-methylnitrosourea	++++	−	−
129/J	γ-rays	NA[d]	NA	NA
	N-methylnitorsourea	++	++	++
Number analyzed, 58 tumors		28	17	13

[a]NIH 3T3 transformants were obtained in the focus forming assay or the nude mouse assay. DNAs from transformants were screened for specific K- and N-*ras* sequences by Southern blot analysis and by oligonucleotide mismatch hybridization.

[b]NIH 3T3 transformants negative for *ras* transforming sequences were screened with a panel of known oncogene probes.

[c]Frequency distribution of *ras* and non-*ras* transforming sequences in NIH 3T3 transformants. Number of transformants identified per total number of positive transformants analyzed: (+) 0-25%; (++) 26-50%; (+++) 51-75%; (++++) 76-100%.

[d]NA, not applicable. 129/J mice are resistant to tumor induction.

Analysis of Genetic Mutations in Activated *ras* Oncogenes

The *ras* gene point mutations were identified by screening the DNA from the 3T3 transformants using the oligonucleotide mismatch hybridization method. A total of 45 *ras* positive 3T3 transformants were analyzed and point mutations were identified in 30. Table 2 summarizes these results. Of 28 K-*ras* positive 3T3 transformants, 21 (75%) contained the G to A transition previously identified in codon 12 of the K-*ras* gene (*15*). This mutation substitutes aspartate (GAT) for glycine (GGT). The remaining 7 K-*ras* positive transformants contain a different mutation(s) which has yet to be identified.

The mutations occurring in the N-*ras* gene, although less frequently activated than the K-*ras* gene, were much more heterogeneous in their nature. A total of 17 N-*ras* positive transformants were analyzed. Mutations were identified in 9 (53%) transformants, which affected all three codons (12, 13, and 61).

Previously we have identified a CAA to AAA transversion in the 61st codon of the N-*ras* gene that substitutes glutamine for lysine (*16*). One-third of the N-*ras* positive transformants contained this same mutation. An additional mutation was localized to codon 61 by cloning and sequencing (*10*). This was a CAA to CTA mutation changing glutamine for leucine.

Approximately half of the mutations occurred as GGT to GAT transitions in codon 12 (4 transformants) and in codon 13 (1 transformant). These mutations result in the substitution of aspartate (GAT) for glycine (GGT).

Unknown genetic factors appear to influence which *ras* gene becomes mutated, as well as the frequency with which specific codons are mutated. NMU treatment of C57BL/6 or (AKR × RF)F_1 mice results in preferential activation of the K-*ras* and N-*ras* genes respectively. As shown in Table 2, the treatment of RF/J strain mice with two different agents, γ radiation and the chemical NMU, results in identical point mutations in the 12th codon of the K-*ras* gene or the N-*ras* gene, arguing against carcinogen specific mutations in this mouse strain.

The K-*ras* gene is most frequently identified as the transforming sequence in carcinogen-induced tumors of all the strains of mice tested so far. The overwhelming majority of the transformants contain the identical mutation in codon 12, a GGT to GAT transition, irrespective of the inducing agent used.

Somatic Loss of Normal *ras* Allele in Carcinogen-Induced Thymic Lymphomas

Homozygosity of abnormal genes is a frequently observed phenomenon in several human malignancies (*17–19*). We have reported loss of the normal N-*ras* allele in one (AKR × RF)F_1 NMU-induced thymic lymphoma (*20*).

We therefore examined the allelic composition of some of the DNAs from carcinogen-induced thymic lymphomas that transferred activated K-*ras* genes. Using oligonucleotide mismatch hybridization with oligomers that recognize the normal and the mutated DNA sequences, primary tumors can be screened for the dosage of the allele variants. Four tumors analyzed to date show homozygosity for the abnormal K-*ras* allele (*10*). Thus, it appears that tumors with activated *ras* genes have a tendency to lose the normal copy and/or increase the number of copies of the mutant allele (Fig. 2). This might be one of the steps involved in tumor progression.

A working model of the changes that may occur *in vivo* in *ras* genes following carcinogen exposure is shown in Figure 2. Initiation, the exposure to carcinogenic agents, may result in mutation of the K- or N-*ras* genes directly or indirectly through the action of other genes. Mutations that are relevant biologically, i.e., confer a growth advantage to a cell, are selected *in vivo*. During tumor progression the mutated allele undergoes duplication. Subsequently, in some percentage of the tumors, the normal *ras* allele is lost.

Conclusions and Perspectives

Inbred strains of mice have been treated with different carcinogenic agents, gamma or neutron radiation, and *N*-nitrosomethylurea. These agents induce thymic lymphomas with high frequency. Activation of oncogenes of

Table 2. Frequency distribution of point mutations identified in activated *ras* oncogenes by mismatch hybridization.

Strain of mouse	Treatment of animal	Characterization of mutation					
		K-*ras*		N-*ras*			
		codon 12	Unknown[a]	12	13	61	Unknown[a]
(AKR × RF)F_1	γ-rays	+++[b]	+	–	–	–	–
	N-methylnitrosourea	–	–	+	–	+	+++
RF/J	γ-rays	+++	+	+	–	–	–
	N-methylnitrosourea	+++	+	+	–	+++	–
C57BL/6J	γ-rays	+	++	++			
	N-methylnitrosourea	++++	–	–			
129/J	γ-rays	NA[c]	NA	NA	NA	NA	NA
	N-methylnitrosourea	+++	–	++	++	–	–
Number analyzed, 45 tumors		21	7	4	1	4	8

[a]Unknown mutation.

[b]Frequency distribution of *ras* mutations in *ras*-positive NIH 3T3 transformants. Number of K- or N-*ras* mutations identified per total number of K- or N-*ras* positive transformants analyzed: (+) 0–25%; (++) 26–50%; (+++) 51–75% (++++) 76–100%.

[c]NA, not applicable. 129/J mice are resistant to tumor induction.

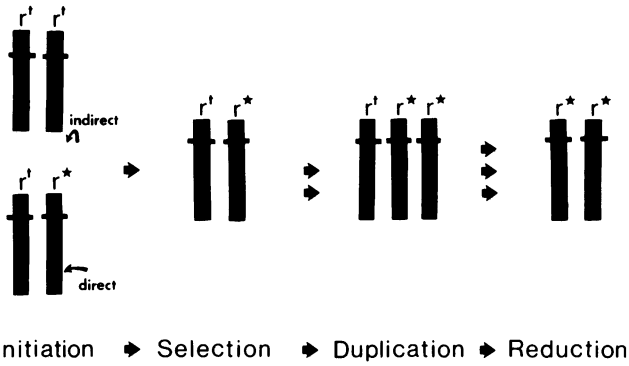

FIGURE 2. A model of the role that *ras* genes may play at different stages of carcinogen-induced disease.

the *ras* gene family have been systematically surveyed in these tumors. The main conclusions from these studies are:

a) The genetic background of the host modifies the susceptibility of a particular mouse strain to the action of a particular carcinogen (i.e., 129/J mice are resistant to γ irradiation).
b) The only *ras* oncogenes detected are K-*ras* and N-*ras*.
c) Oncogene activation is not carcinogen specific (i.e., the same carcinogen activates different *ras* genes in different mouse strains) but is modified by unknown host genes.
d) Activation of the K-*ras* gene occurs predominantly by a point mutation in codon 12 resulting in a GGT to GAT transition.
e) Activation of the N-*ras* gene occurs by point mutations in codons 12, 13, or 61.
f) Somatic loss of the normal N-*ras* or K-*ras* gene together with duplication of the mutated *ras* gene occurs during tumor progression.

Our previous studies have used protocols requiring multiple exposure of the animals to the carcinogen. More recently, C57BL/6J, RF/J and 129/J mice were treated with a single exposure to NMU (80 mg/kg). Similarly, RF/J mice received a single exposure to neutron radiation. Thymic lymphomas were induced in NMU-treated C57BL/6J mice and neutron-irradiated RF/J mice. We want to examine the frequency of oncogene activation in these tumors and identify the mutations. In addition, we hope to be able to determine more precisely the timing of the events outlined in Figure 2 that occur in high frequency in *ras* genes in this model system of carcinogen-induced murine thymic lymphoma.

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